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GRANT NUMBER DAMD17-94-J-4179

TITLE: Her-2/Neu and Breast Cancer

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REPORT DATE: August 1997

TYPE OF REPORT: Annual

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	August 1997	Annual (8 Jul 96 - 7 Jul 97)

4. TITLE AND SUBTITLE
Her-2/Neu and Breast Cancer

5. FUNDING NUMBERS
DAMD17-94-J-4179

6. AUTHOR(S)
Joni Doherty
Doctor Gail Clinton

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Oregon Health Sciences University
Portland, Oregon 97201-3098

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 2170-25012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200*

Overexpression of the HER-2/*neu* proto-oncogene as a 185 kDa transmembrane tyrosine kinase occurs in approximately 30% of human breast and ovarian cancers and is associated, in a dose-dependent manner, with poor prognosis for survival of the patient. Although HER-2 gene amplification is a common mechanism for overexpression, many human tumors and cell lines display HER-2 mRNA and protein overexpression in excess of the level accountable by gene copy number, or in absence of gene amplification. Thus, mechanisms of transcriptional and post-transcriptional upregulation occur with respect to HER-2. Such mechanisms require further investigation with respect to human tumors and cell lines. Notably, the highest HER-2 protein-overexpressing cell line, SK-OV-3, exhibits one of more of these mechanisms in addition to gene amplification. Furthermore, in addition to overexpression of the normal-sized 4.5 kb HER-2 mRNA transcript, SK-OV-3 also expresses an abundant alternative HER-2/*neu* transcript of 8 kb--the focus of my current research. Preliminary data demonstrates that this aberrant 8 kb transcript displays increased mRNA stability, and may have an altered 3'UTR sequence. The present work focuses on characterizing this transcript in terms of (1) structure/sequence, (2) origin, (3) mechanism(s) for accumulation in the cell, and (4) function.

14. SUBJECT TERMS
Breast Cancer HER-2/*neu*, proto-oncogene, 3'UTR, 5'UTR, RT-PCR, RACE, $p185^{HER\text{-}2/neu}$

15. NUMBER OF PAGES
21

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

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Jay K. Doherty 8-4-97

PI - Signature Date

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Characterization of an Alternative HER-2/*neu* mRNA Transcript

INTRODUCTION

Nature of the Problem and Background of Previous Work

The HER-2/*neu* (c-erbB-2) is developmentally expressed in many tissues as a 185 kDa transmembrane tyrosine kinase ($p185^{HER\text{-}2/neu}$) with extensive homology to the Epidermal Growth Factor Receptor (EGFR) (1). $p185^{HER\text{-}2/neu}$ has been shown to signal through the mitogen-activated protein (MAP) kinase pathway, inducing nuclear events that may lead to proliferation and growth of tumor cells (2). Like EGFR, overexpression of HER-2/*neu* has been reported in an array of human carcinomas (3), most notably in up to 30% of breast and ovarian cancers. Moreover, HER-2/*neu* overexpression is associated with increased tumor invasiveness, malignancy, and poor prognosis for survival of the patient (4).

HER-2/*neu* (erbB-2) is the human homologue of the rat *neu* oncogene, which was first identified in rat nervous system tumors induced by N-ethyl-N-nitrosurea (EtNU). Transforming rat *neu* has been shown to be "activated" by a point mutation resulting in Valine to Glutamate transition at residue 664 in TM domain. This activated form of *neu* shows increased tyrosine kinase activity. Surprisingly, no such mutations have yet been identified in human HER-2/*neu*. (3, 5) Transfection of the human HER-2/ *neu* cDNA into NIH-3T3 cells is alone transforming when overexpressed. Implantation of such HER-2-transformed NIH-3T3 cells into nude mice leads to rapid tumor formation. Wild type human HER-2/*neu,* under regulation of the MMTV-LTR promoter/enhancer, induces mammary tumors in transgenic mice (6). Moreover, several lines of evidence indicate that blocking HER-2/neu overexpression reverts tumor growth (). Thus, studies indicate that HER-2/ *neu* overexpression is an important and common mechanism for tumor formation and progression.

The mechanisms for $p185^{HER\text{-}2/neu}$ overexpression have not been entirely elucidated, although HER-2/*neu* gene amplification has been shown (3, 5, 7). However, many tumors and cell lines display overexpressed HER-2/ *neu* mRNA and protein, in absence of gene amplification (3, 7, 8). Although mechanisms for gene amplification and transcriptional upregulation are not well understood, studies suggest selection for $p185^{HER\text{-}2/neu}$ overexpression in some human breast and ovarian cancers and cell lines (3, 7, 8).

As a general rule, cancer cells display genetic instability resulting in mutations, gene amplification, deletion, and rearrangement. Such events may provide additional mechanisms for HER-2/*neu* overexpression to occur that have not yet been revealed (3). Possible mechanisms for overexpression include: (a) increased gene copy number, (b) increased rate of transcription initiation, (c) increased HER-2/*neu* messenger RNA transcript stability, (d) enhancement of translation efficiency from altered HER-2/*neu* mRNA transcript, and (e) decreased turnover of protein product.

Increased transcription initiation rate could be due to (1) alterations in transcription factors, (2) altered transcription factor availability, or (3) change in gene regulatory sequences, such as promoter, silencer, repressor, or enhancer mutations. Aberrant genetic events that have been identified in human cancers can involve translocations that result in gene transcription under the control of an alternative gene promoter (9). Such events could result in HER-2 overexpression, under the control of a strong promoter, and gene duplication might even lead to tandem repeats of the HER-2/*neu* gene.

Increased mRNA stability can result from mutational changes affecting: (1) the length of the 3'untranslated region (3'UTR), (2) sequences within the 3'UTR that confer rapid degradation, and (3) binding sites for stabilizing proteins. Possible targets capable of having such effects if mutated include: AU-rich sequences in the 3'UTR (10), deadenylation signals, decapping signals (11), affinity binding sites for poly-A-binding protein (PAB) that may stabilize mRNA and also enhance translational efficiency (12), and altered stem-loop structures in 5'UTR and/or 3'UTR that may affect degradation rate (11-13).

Posttranscriptional mechanisms of overexpression due to an altered mRNA sequence might include translational upregulation or decreased protein turnover rate. Translational efficiency can be affected by changes in mRNA sequence, including both 5'UTR and 3'UTR (13, 14) and base pair mutations in signal sequences within the coding region that would normally induce cotranslational degradation (15). Additionally, sequence changes that lead to amino acid substitution might result in (a) stabilizing the translated HER-2 protein, or (b) its overexpression at the cell surface by affecting its internalization rate.

Mechanisms for overexpression of p185$^{HER\text{-}2/neu}$ involving sequence variations in the HER-2 gene and within its mRNA transcript require further investigation.

Alternative mRNA transcripts for the EGFR, suggested to confer enhanced oncogenicity, have been identified in human brain tumors and cancer cell lines (4, 9, 16). Likewise, alternative HER-2/*neu* mRNA transcripts have, in fact, been identified in both human tumors and tumor cell lines (7, 17). Of interest, the SK-OV-3 human ovarian cancer-derived cell line, which produces the highest level of p185$^{HER\text{-}2/neu}$, displays an abundant mRNA

species of approximately 8 kb, in addition to the well-characterized and cloned 4.5 kb HER-2/*neu* transcript normally seen.

SK-OV-3 is a good model for revealing mechanisms of HER-2/*neu* overexpression because it displays (a) HER-2/*neu* gene amplification with, reportedly, eight copies of the gene, (b) evidence for gene rearrangement with restriction fragment length polymorphism in the genomic HER-2 sequence, and (c) an abundant alternative HER-2/*neu* transcript that has not yet been characterized. Furthermore, SK-OV-3 display an agressive and invasive phenotype and are frequently used as an in vivo model for tumor metastasis and effects of anti-HER-2 therapy. In fact, Yu et al. () reported that when SK-OV-3 cells were selected for a more metastatic phenotype in nude mice, they showed upregulation of $p185^{HER\text{-}2/neu}$, without any further gene amplification. Thus, SK-OV-3 studies may provide information elucidating the role of HER-2 overexpression in tumor progression to a more metastatic state.

The fact that this alternative transcript appears to be selected for in this aggressive cancer cell line suggests that it may be contributing to tumorigenesis. One possibility is that this alternative transcript may facilitate overexpression of HER-2/*neu* , thus contributing to tumorigenesis; however, other possibilities for its selection in these cells may exist. In order to determine the function of this alternative transcript, it will be necessary to determine its sequence and characterize its translation product. The proposed study is thus aimed at structurally and functionally characterizing this alternative 8 kb HER-2/*neu* transcript in SK-OV-3 cells, in terms of identifying sequence alterations that potentially contribute to $p185^{HER\text{-}2/neu}$ overexpression, activation, and tumorigenicity.

Purpose of the Present Work

The purpose of my current thesis project, as a DOD predoctoral training fellowship recipient, is to elucidate the role of an alternative mRNA transcript for HER-2/neu with respect to $p185^{HER\text{-}2/neu}$ overexpression in human breast and ovarian cancers.

BODY

Experimental Methods, Assumptions and Procedures

Based on preliminary data (described in my 1996 annual report) and previous reports (17), I have proceeded with the characterization of the aberrant 8 kb HER-2 transcript under the assuption that it is translated into functional $p185^{HER\text{-}2/neu}$. Furthermore, I have begun to collect evidence that further supports this assumption.

8 kb HER-2/neu Transcript, Sequence Content

Northern blotting experiments are performed using SK-OV-3 total RNA, extracted via Triazole, or poly-A+ RNA, selected by oligo-dT column elution. Blots are hybridized with various HER-2 cDNA region-specific probes to study the hybridization efficiency of the 8 kb HER-2 transcript as compared to the normal 4.5 kb transcript in SK-OV-3. Northern blots are analyzed by phosphorimager, using IPLab Gel (Molecular Dynamics) to quantitate bands.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) is used to directly obtain alternative sequence information from the 8 kb HER-2 transcript. Purification of SK-OV-3 mRNA and subsequent size fractionation is required in order to study the 8 kb HER-2 transcript independently of the normal-sized 4.5 kb transcript. Fractionated mRNA is reverse-transcribed into first strand cDNA, which is used as a template for amplification via the polymerase chain reaction using HER-2 sequence-specific primers. Electrophoresis of RT-PCR products reveals size of the region amplified; larger products than those obtained from the 4.5 kb fraction are then purified for restriction enzyme digest, subcloning, and sequencing.

8 kb HER-2/neu , transcript stability

SK-OV-3 cultures were treated with transcriptional inhibitors for varying times prior to extraction of total RNA for Northern blotting experiments to determine the relative mRNA stability of the 8 kb HER-2 transcript compared to the 4.5 kb transcript and previously reported results.

Results and Discussion

Northern blotting of cellular RNA

To determine whether the "wild-type" HER-2/*neu* sequence is contained within the larger, 8 kb HER-2/*neu* transcript from SK-OV-3 cells, I performed Northern blotting of SK-OV-3 cell mRNA, hybridizing with both 5' HER-2 coding and 3'UTR sequence, as random prime-labeled cDNA probes, and also with 3' HER-2 coding antisense RNA sequence (see figure 1, Appendix). Hybridization with these probes, shows an approximately equal distribution ratio between 8 kb and 4.5 kb transcripts, suggesting that the entire HER-2 coding sequence is contained within this aberrant transcript. However, these data do not rule out

the existence of insertions or interruptions of the HER-2 coding sequence. Hybridization efficiency ratios were quantified by phosphorimager analysis of three separate experiments (using dot blots to control for probe specific activity, data not shown).

HER-2 mRNA stability determination

Because Northern blotting experiments reveal greater abundance of the 8 kb HER-2 mRNA transcript, as compared to the 4.5 kb (fig. 1), I have begun to investigate mechanisms for transcript accumulation. Possibilities include either increased transcription initiation rate or increased mRNA stability. Because I have not yet obtained alternative sequence information, I cannot yet assess transcription intiation rate, which would require an RNase protection assay, including a probe comprising some of the alternative sequence contained within the aberrant 8 kb transcript in order to differentiate between 8 kb and 4.5 kb HER-2 transcripts (please see specific aim #3 under "Future Work", page 20).

Therefore, I have compared the relative stability of 8 kb and 4.5 kb HER-2 mRNAs in SK-OV-3. To assess mRNA decay, I conducted separate experiments, treating several 10 cm plates of confluent SK-OV-3 cell cultures with two different transcriptional inhibitors: (a) actinomycin D at 5 μg/ml, and (b) α-amanitin at 24 μg/ml. Total RNA was extracted from duplicate plates at one to six hour time intervals over a period of 33 and 26 hours for each transcriptional inhibitor experiment, respectively. RNA samples were electrophoresed (10 μg/lane of these RNA samples, alongside an RNA sample from identical, but untreated, SK-OV-3 cell culture plates for "time 0" control), Northern blotted and probed with both an 18S riboprobe, to normalize for RNA loading, and an α(^{32}P)dCTP- random prime-labeled HER-2 cDNA probe, to detect the HER-2 mRNA transcript. Figure 2a (see Appendix) shows a representative Northern blot of this method. Relative amounts of 8 kb and 4.5 kb HER-2 mRNA were quantitated by phosphorimager analysis. In figure 2c, the relative mRNA decay rates are shown. The plot in figure 2c represents the average values of initial time points of decay for each transcript from three repeats of Northern blotting RNA from α-amanitin treated cells (as described above), with error bars indicating the variance between data points. Results indicate a longer mRNA half-life for the 8 kb (15 h) as compared to the 4.5 kb (6 h) HER-2 transcript in SK-OV-3 (fig. 2c). The value of 6 h for the 4.5 kb mRNA half-life coincides with previously reported HER-2 mRNA stability studies (18). Furthermore, the difference between these two calculated half lives is statistically significant ($p = 0.01$). This suggests that much of the overexpression of this aberrant 8 kb HER-2 transcript may be due to accumulation in the cell because of increased mRNA stability.

Reverse Transcription-Polymerase Chain Reaction

Many studies have indicated a role for the 3'UTR in mRNA stability, either varying with length or the presence of consensus sequences that confer rapid mRNA degradation (10-15); therefore, I am investigating the possibility that a difference in 3'UTR in the 8 kb HER-2 transcript may exist. To isolate the 8 kb

from the 4.5 kb HER-2 transcripts, I modified the method of electroelution from agarose gels described by O'Callaghan et al. (1991, ref. 19). I electroeluted these mRNA transcripts by size from denaturing (formaldehyde-containing) 0.7 % agarose-MOPS gels and subsequently dialyzed away the formaldehyde, reverse transcribed the mRNA and use this as a template for polymerase chain reactions.

To investigate sequences in the 3'UTR of these HER-2 transcripts, I performed 3'Rapid Amplification of cDNA Ends (3'RACE), using a nested PCR strategy. An oligo-dT was used as a downstream (antisense direction) primer (i.e., to amplify from the poly-A tail) and HER-2 specific upstream (sense direction) outer and inner (with an EcoR1 restriction site sequence added to the 5'end to facilitate subcloning) primers were used in sequential rounds of the PCR. Figure 3(a) shows a schematic of this method, indicating that an amplification product of about 650 bp is expected from the 'normal' 4.5 kb transcript and from an HER-2 expression vector control plasmid (p9002, containing the full-length HER-2 cDNA cloned from a human placental cDNA library).

The left panel of Figure 3(b) shows a representative agarose gel on which I have run final HER-2 3'RACE amplification products of: (1) no DNA (control); (2) p9002 (HER-2 expression vector showing a faint 650 bp band as expected from a 1:300 dilution of the outer reaction in a nested PCR; (3) reverse transcribed mRNA from T47D--a human breast carcinoma cell line that does not express an aberrant HER-2 transcript and does not exhibit HER-2 gene amplification nor rearrangement; (4) reverse transcribed SK-OV-3 mRNA from the 8kb fraction; and (5) 4.5 kb fraction, showing an abundant amplification product at 650 bp as expected for the 'wild-type' transcript. The right panel of figure 3(b) shows the southern blot of this gel, hybridized with a HER-2 sequence specific probe within the region to be amplified (i.e., just 3' to the inner PCR primer). Repeating this experiment numerous times with varying PCR conditions yielded no HER-2-specific products for the 8 kb transcript in 3'RACE RT-PCR. These results can be interpreted to indicate either that (a) the 3'UTR of the 8 kb transcript is different from that of the 4.5 kb, or (b) the 3'RACE PCR strategy I have used does not amplify a product from this transcript due to (i) complex secondary structure that inhibits primer annealing and/or elongation, (ii) primer selection is poor for this transcript due to a mutation at the primer annealing site in the 8 kb transcript, or (iii) the region is too long to be efficiently amplified by the PCR. Therefore, I am currently attempting an alternative strategy, shown schematically in figure 4a, in which I prime the reverse transcription (RT) with a mixture of random hexamers that are linked to a specific sequence of nucleotides containing a HindIII restriction site. Subsequently this material is used as a template for 3'RACE using a downstream primer that is specific to the sequence of nucleotides containing the HindIII site. I can, thus, amplify with the same HER-2-specific nesting primers and obtain products that can be digested with EcoR1 and HindIII for subcloning into m13 and sequencing the 3'UTR of the 8 kb HER-2 transcript. Figure 4 shows a representative gel (b) with corresponding Southern blot (c), hybridized with the same oligonucleotide probe as described above.

Finally, in order to determine whether the 8 kb transcript contains the same length of coding sequence as the wild-type or 4.5 kb, I amplified a 3.6 kb region of the coding sequence by PCR. Figure 5a shows a representative gel on which I electrophoresed amplification products from no DNA (control), p9002, the 8 kb fraction of cDNA from SK-OV-3, and 4.5 kb fraction. All three of these HER-2 sequence-containing templates show amplification of the 3.6 kb coding region, as expected. A Southern blot of the gel in 5a shows that all three of these 3.6 kb bands hybridize with an $\alpha(^{32}P)$dCTP- random prime-labeled HER-2 cDNA probe (fig 5b).

To determine the 3'UTR and other unique sequence of an aberrant 8 kb HER-2/neu mRNA transcript expressed in SK-OV-3 cells and to clone its cDNA form, I continue to perform: (A) amplification via polymerase chain reaction, with the goal of subsequent subcloning and sequencing. Reverse transcription (to form cDNA) coupled with polymerase chain reaction (RT-PCR) using specific primers targeting amplification of the 3'UTR and 5'UTR of the 8 kb HER-2/ *neu* transcript cDNA sequence; and (B) subcloning and sequencing of relevant PCR products (i.e., those that hybridize with HER-2 probes upon Southern blotting, indicating that they contain relevant HER-2 sequence). Purified PCR amplification products will be restriction digested, rerun on a DNA-preparatory agarose gel, excised according to size, and eluted for ligation into pSK (Bluescript) vectors, and directly sequenced using pSK-specific primers.

Novel sequence obtained through the RT-PCR and subcloning experiments described is entered in the database GenBank and compared for sequence homology with any currently known segments of DNA or RNA that may elucidate the functional significance and origin of the alternative transcript sequence.

Potential difficulties in this methodology that may lead to artifactual PCR products and/or misleading results include contamination of the 8 kb mRNA fraction with molecules of 4.5 kb transcripts due to the nature of the isolation technique. This might result in preferential amplification of the smaller transcript. Another potential obstacle would be if the larger HER-2 transcript has complex secondary structure that inhibits reverse transcription, and possibly even inhibits polymerase elongation, making it difficult to amplify.

CONCLUSIONS

Implications of Completed Research

My preliminary results suggest that (1) the 8 kb transcript exhibits increased mRNA stability in SK-OV-3 cells, (2) the 3'UTR of this transcript may be different from that of the 4.5 kb, and (3) the length of its coding region and ratios between mRNA and protein overexpression in SK-OV-3 cells suggest that this 8 kb transcript is translated into p185$^{HER-2/neu}$.

Future Work

Specific Aim #1. I will obtain alternative sequence information contained within the larger, aberrant 8 kb HER-2 transcript. If I cannot obtain the aberrant sequence via RT-PCR, I will determine the sequence of the alternative transcript sequence by screening λgt10 partial cDNA library. To do this, I will reverse transcribe only the 8 kb size-fractionated, purified mRNA in order to form cDNA. I will ligate this cDNA with adapters and clone into λgt10. I will then directly sequence all clones that hybridize with a full-length HER-2 cDNA probe. Next, I will generate full-length cDNA clones of the aberrant 8 kb HER-2 mRNA transcript by restriction mapping of partial cDNA cloned sequences and ligate this full-length cDNA into a pUC8 vector for further analysis.

In order to assess whether the 8 kb mRNA is translated and to characterize the protein product of this alternative transcript, I will subclone the full-length 8 kb cDNA into an appropriate expression vector for in vitro transcription-translation experiments, using the p9002 HER-2 expression vector as a control. I will first standardize the amount of protein translated to the level of transcript synthesized in vitro by incorporating a(32P)CTP into in vitro transcription reaction and electrophoresing products on a denaturing agarose gel, which will be exposed to autoradiography.

Specific Aim #2. I will determine whether this alternative HER-2/neu transcript is a product of gene rearrangement or alternative splicing. By PCR, using primers that flank the point of divergence of the aberrant sequence in the 8 kb cDNA, I will amplify the encompassing region from both SK-OV-3 genomic DNA and control DNA, from a cell line that does not express an aberrant HER-2 transcript nor exhibits gene rearrangement (e.g., T47D) and electrophorese the PCR products. If the aberrant transcript is a result of alternative splicing, then I expect the sequence to be contiguous in the genome, due to read-through of a consensus splice site. Therefore, I expect the PCR to amplify the same sized fragment from both SK-OV-3 and from genomic DNA extracted from any cell line or tissue (given that it does not have a mutation or rearrangement at this site). Conversely, if this aberrant transcript results from a gene rearrangement, then I expect to amplify the divergent sequence directly from only SK-OV-3 genomic DNA. However, this may not be an effective strategy if, for example, the region I choose to amplify by PCR contains more than a single intron-exon junction in the genome, and intron are too large to be amplified efficiently by the PCR. Obtaining sequence information about the divergent region in the 8 kb transcript will facilitate the design of this experiment.

Specific Aim #3. To test the possibility that additional accumulation of the aberrant 8 kb HER-2/*neu* mRNA in SK-OV-3 cells is due to enhanced transcriptional rate, I will perform transcription initiation rate assessment via nuclear run-on assays. To perform these studies, I will first need to know at least a portion of the alternative sequence of the 8 kb in order to synthesize transcript-specific probes to distinguish between the 4.5 kb and 8 kb transcript

in an RNase Protection assay. Briefly, this assay will consist of prolonged hybridization of HER-2 transcript-specific riboprobes with in vitro synthesized total RNA purified as nuclear exports from isolated SK-OV-3 nuclei after cell lysis in non-ionic buffer (containing NP-40). The hybridization reaction will then be subjected to RNase H digestion, which will spare only double stranded RNA-riboprobe hybrids, which will be electrophoresed on a nondenaturing gel and subjected to autoradiography. The two transcript-specific probes used will be sufficiently different in size to distinguish the hybrids by this method. A control riboprobe that will hybridize with a ubiquitously expressed mRNA, such as GAPDH or 18S, should also be added to the reaction. Alternatively, unlabelled probe DNA sequences can be denatured and immobilized on a nylon membrane (in excess) at specific locations. Isolated nuclei would be treated with radiolabeled nucleotides to attain radiolabeled total RNA synthesis in vitro. This radiolabeled RNA would then be hybridized with the nylon membrane, which would subsequently be washed to remove non-hybridizing RNAs, and exposed to autoradiography. Both of these methods could be used for quantification of relative transcript initiation rates by phosphorimager analysis, but the former method is probably more sensitive.

References

1. Coussens, L., Yang-Feng, T.L., Liao, Y.-C., Chen, E, Gray, A., McGrath, J., Seeburg, P.H., Libermann, T.A., Schlessinger, J., Francke,U., Levinson, A., and Ullrich, A. Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with neu oncogene. *Science* 230: 1132-9, 1985.

2. Ben-Levy, R., Paterson, H.F., Marshall, C.J., and Yarden, Y. A single autophosphorylation site confers oncogenicity to the neu/erbB-2 receptor and enables coupling to the MAP kinase pathway. *The EMBO Journal* 13(14): 3302-11, 1994.

3. Hynes, N. E. and Stern, D. F. The biology of erbB-2/neu/HER-2 and its role in cancer. *Biochimica et Biophysica Acta* 1198: 165-84, 1994.

4. Hung, M.-C., Zhang, X., Yan, D.H., Zhang, H.-Z., He, G.-p., Zhang, T.-q., and Shi, D.-r. Aberrant expression of the c-erbB-2/neu protooncogene in ovarian cancer. *Cancer Letters* 61: 95-103, 1992.

5. Kanai, Y. Ochiai, A., Shibata, T., Oyama, T., Ushijima, S., Akimoto, S., and Hirohashi, S. *C-erbB-2* gene product directly associates with β-catenin and plakoglobin. *Biochemical and Biophysical Research Communications* 208(3): 1067-72, 1995. Ochiai, A., Akimoto, S., Kanai, Y., Shibata, T., Oyama, T., and Hirohashi, S. *c-erbB-2* gene product associates with catenins in human cancer cells. *Biochemical and Biophysical Research Communications* 205(1): 73-8, 1994.

6. Shibata, T., Ochiai, A., Kanai, Y., Akimoto, S., Gotoh, M., Yasui, N., Machinami, R., and Hirohashi, S. Dominant negative inhibition of the association between β-catenin and c-erbB-2 by N-terminally deleted β-catenin suppresses the invasion and metastasis of cancer cells. *Oncogene* 13: 883-9, 1996.

5. Slamon, D.J., Godolphin, W., Jones, L.A., Holt, J.A., Wong, S.G., Keith, D.E., Levin, W.J., Stuart, S.G., Udove, J., Ullrich, A., and Press, M.F. Studies of the HER-2/neu proto-oncogene in human breast and ovarian cancer. *Science* 24: 707-12, 1989.

6. Muller, W.J., Sinn, E., Pattengale, P.K., Wallace, R., and Leder, P. Single step induction of mammary adenocarcinoma in transgenic mice bearing activated c-*neu* oncogene. *Cell* 54: 105-115, 1988.

7. Tyson, F.L., Boyer, C.M., Kaufman, R., O'Briant, K., Cram, G., Crews, J.R., Soper, J.T., Daly, L., Fowler, W.C., Jr., Haskill, J.S., and Bast, R.C., Jr. Expression and amplification of the HER-2/neu (c-erbB-2) protooncogene in epithelial ovarian tumors and cell lines. *American Journal of Obstetrics & Gynecology* 165(3): 640-6.

8. Lonn, U., Lonn, S., Nilsson, B., and Stenkvist, B. Intratumoral heterogeneity for amplified genes in human breast carcinoma. *Internal Journal of Cancer* 58: 40-5, 1994.

9. Vu, T.H., Martin, G., Lee, P., Mark, D., Wang, A., and Williams, L.T. Developmentally regulated use of alternative promoters creates a novel platelet-derived growth factor receptor transcript in mouse teratocarcinoma and embryonic stem cells. Molecular and Cellular Biology 9(10): 4563-7, 1989.

10. DeMaria, C.T., & Brewer, G. AUF1 binding affinity to A+U-rich elements correlates with rapid mRNA degradation. The Journal of Biological Chemistry 271(21): 12179-84, 1996.

11. Beelman, C.A. & Parker, R. Degradation of mRNA in eukaryotes. Cell 81: 179-83, 1995.

12. Jackson, R.L. & Standart, N. Do the poly(A) tail and 3'untranslated region control mRNA translation? Cell 62: 15-24, 1990.

13. Sonnenburg, N. mRNA translation: Influence of the 5' and 3' untranslated regions. Current Opinions in Genetics and Development 4: 310-15, 1994.

14. Tanguay, R.L. and Gallie, D.R. Translational efficiency is regulated by the length of the 3' untranslated region. *Molecular and Cellular Biology* 16(1): 146-56, 1996.

15. Sachs, A.B. Messenger RNA degradation in eukaryotes. Cell 74: 413-21, 1993.

16. Collins, V.P. Epidermal growth factor gene and its transcripts in glioblastomas.Recent Results in *Cancer Research* 135: 17-24, 1994. Callaghan, T., Antczak, M., Flickinger, T., Raines, M., Myers, M., and Kung, H.-J. A complete description of the EGF-receptor exon structure: implication in oncogenic activation and domain evolution. *Oncogene* 8: 2939-48, 1993. Wong, A.J., Ruppert, J.M., Bigner, S.H., Grzeschik, C.H., Humphrey, P.A., Bigner, D.S., and Vogelstein, B. Structural alterations of the epidermal growth factor receptor gene in human gliomas. *Proceedings of the National Academy of Sciences*, USA 89: 2965-69, 1992. Ekstrand, A.J., Sugawa, N., James, C.D., and Collins, V.P. Amplified and rearranged epidermal growth factor receptor genes in human glioblastomas reveal deletions of sequences encoding portions of the N- and/or C-terminal tails. *Proceedings of the National Academy of Sciences,*USA 89: 4309-13, 1992. Ullrich, A., Coussens, L., Hayflick, J.S., Dull, T.J., Gray, A., Tam, A.W., Lee, J., Yarden, Y., Libermann, T.A., Schlessinger, J., Downward, J., Mayes, E.L.V., Whittle, N., Waterfield, M.D., and Seeburg, P.H. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature* 309: 418-25, 1984.

17. Fujimoto, J., Hori, M., Ichigo, S., Misao, R., and Tamaya, T. Preliminary study of oncogene expressions in endometrial cancers: Aberrant estrogen receptor gene and erbB-2 expressions. *European Journal of Gynaecological Oncology* 16(1): 40-7, 1995. Karlan, B.Y., Jones, J, Slamon, D.J., and Lagasse, L.D. Glucocorticoids stabilize HER-2/neu messenger RNA in human epithelial ovarian carcinoma cells. *Gynecologic Oncology* 53: 70-77, 1994. Jones, J., Lagasse, L.D., and Karlan, B.Y. Steroid hormone independenceof HER-2/neu mRNA expression in four human ovarian cancer cell lines. *Gynecologic Oncology* 55: 421-6, 1994. Scott, G.K, Robles, R., Park, J.W., Montgomery, P.A., Daniel, J., Holmes, W.E., Lee, J., Keller, G.A., Li, W.-L., Fendly, B.M., Wood, W.I., Shepard, M., and Benz, C.C. A truncated intracellular HER2/neu receptor produced by alternative RNA processing affects growth of human carcinoma cells. *Molecular and Cellular Biology* 13(4): 2247-57, 1993. Hung, M-C., Zhang, X., Yan, D-H., Zhang, H-Z., He, G-p., Zhang, T-q., and Shi, D-r. Aberrant expression of the c-erbB-2/neu protooncogene in ovarian cancer. Cancer Letters 61: 95-103, 1992. Lichtenstein, A., Berenson, J., Gera, J.F., Waldburger, K., Martinez-Maza, O., and Berek, J.S. Resistance of human ovarian cancer cells to tumor necrosis factor and lymphokine-activated killer cells: Correlation with expression of HER-2/neu oncogenes. *Cancer Research* 50: 7364-70, 1990. Zhang, X., Silva, E., Gershenson, D., and Hung, M. Amplification and rearrangement of c-erbB proto-oncogenes in cancer of human genital tract. *Oncogene* 4: 985-9, 1989.

18. Karlan, B.Y., Jones, J., Slamon, D.J., and Lagasse, L.D. Glucocorticoids stabilize HER-2/neu messenger RNA in human epithelial ovarian carcinoma cells. *Gynecologic Oncology* 53: 70-7, 1994.

19. Harty, R.N. & O'Callaghan, D.J. Isolation and purification of specific, overlapping mRNA species by electroelution from agarose gels. *Biotechniques* 10(6): 728-9, 1991.

APPENDIX

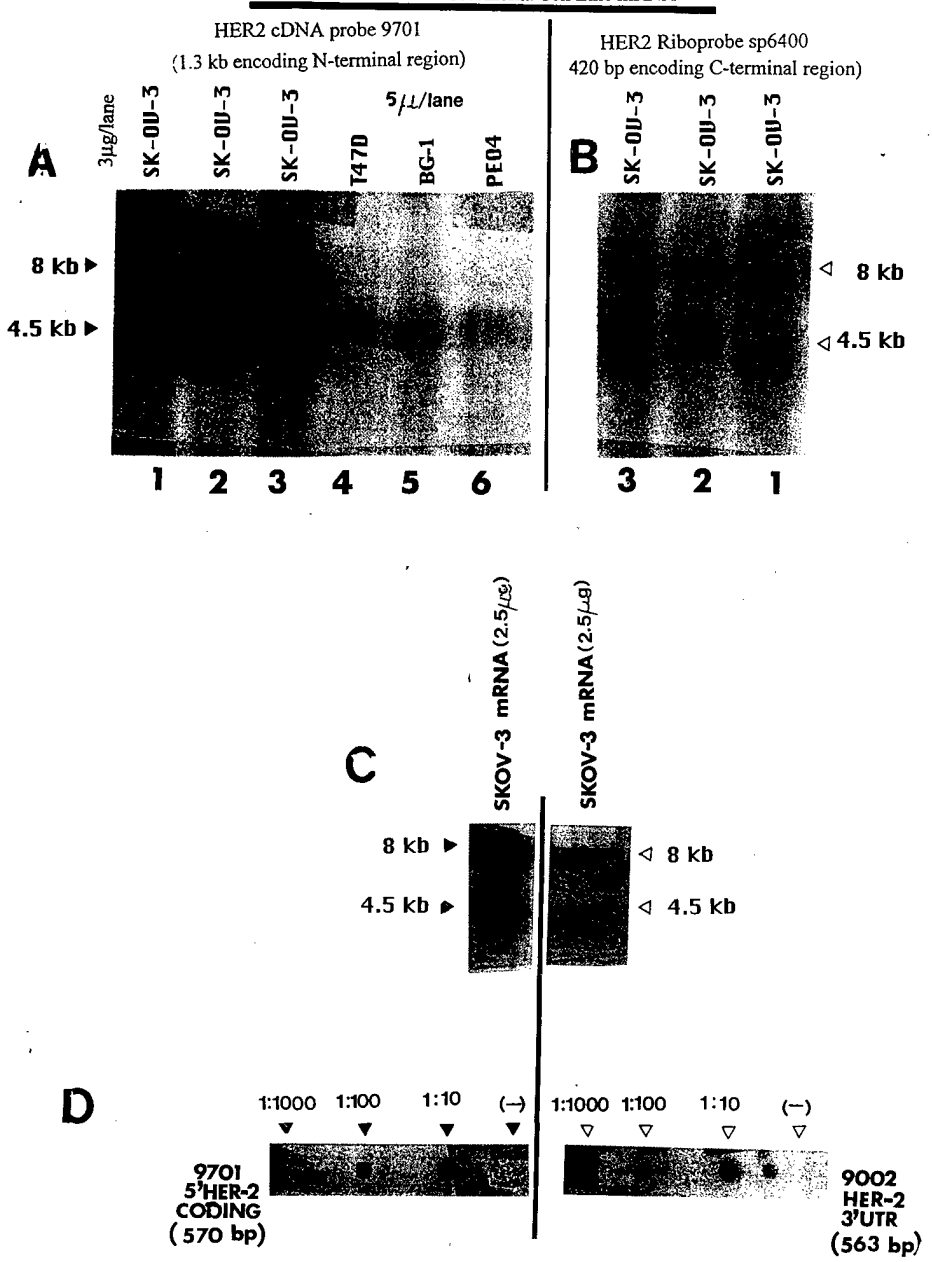
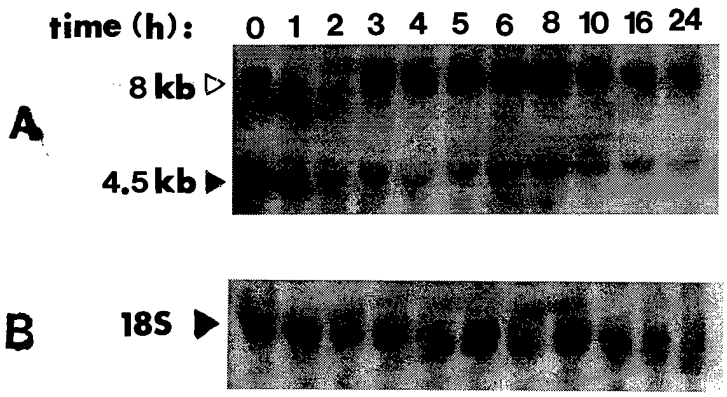


Figure 1. **HER-2/neu probes specific to 5' and 3' coding sequence and 3"UTR hybridize with both the 4.5 kb and aberrant 8 kb HER-2 transcript from SK-OV-3 cells**. Northern blotted mRNAs from three different SK-OV-3 preps (3 μg/lane, lanes 1-3) and the carcinoma cell lines T47D (5 μg, lane 4), BG-1 (5 μg, lane 5), and PEO4 (5 μg, lane 6), probed with a 1.3 kb α(^{32}P)dCTP-labelled probe specific to the 5'HER-2 coding region (9701) which encodes the amino-terminal ligand binding domain of p185$^{HER-2/neu}$ (***A***). Lanes 1-3 of the Northern blot in A were stripped and reprobed with a 420 bp α(^{32}P)CTP-labelled Riboprobe, antisense sequence-specific to the 3'HER-2 coding region (sp6400) corresponding to the c-terminal kinase domain of p185$^{HER-2/neu}$ (***B***). A random-prime-labelled cDNA probe specific to a 563 bp region within the 3'UTR of the 'wild-type' or cloned HER-2 from p9002 hybridizes less efficiently than a 570 bp region of the 5'HER-2 coding sequence (from p9701) probe (***C***) with comparable probe specific activity, varified by 10-fold serially diluted dot-blotted HER-2 expression vector plasmid DNA (p9002), with a 1:10 dilution of vector alone as a negative control (***D***).



HER-2 mRNA decay in SK-OV-3

C

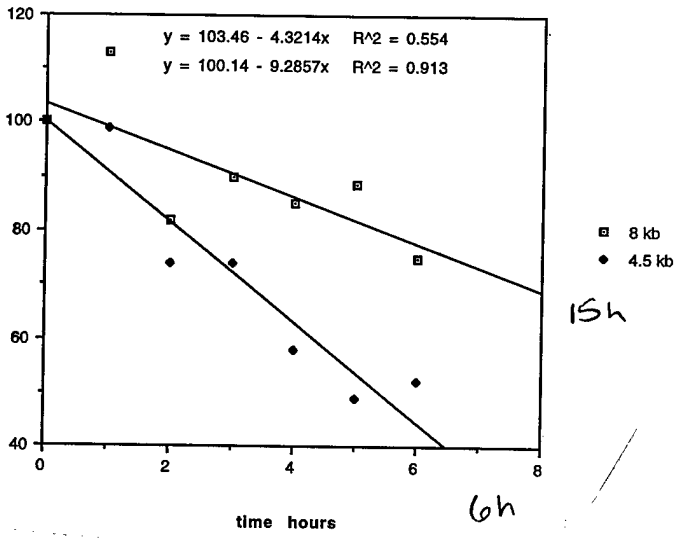


Figure 2. **HER-2/neu mRNA stability in SK-OV-3.** Representative Northern blot of SK-OV-3 total RNA (10 μg/lane) extracted at the indicated time points after α-Amanitin treatment (24μg/ml) of confluent cell cultures in 10 cm plates, probed sequentially with α(^{32}P)dCTP-labelled HER-2 cDNA (***A***) and, to normalize for amount of RNA loaded per lane, 18S Riboprobe (***B***). ***C***: Plot of phosphorimager analysed bands from (***A***) divided by (***B***) (i.e. HER-2 8 kb/ 18S and HER-2 4.5 kb/ 18S), expressed as percent of control (= "time 0") and showing error bars representing variance in data points obtained from three idependent Northern blots of these RNA samples. Calculated mRNA half-lives from the initial decay slope of 8 kb (15 h) and 4.5 kb (6 h) are indicated at the right lower corner in ***C***.

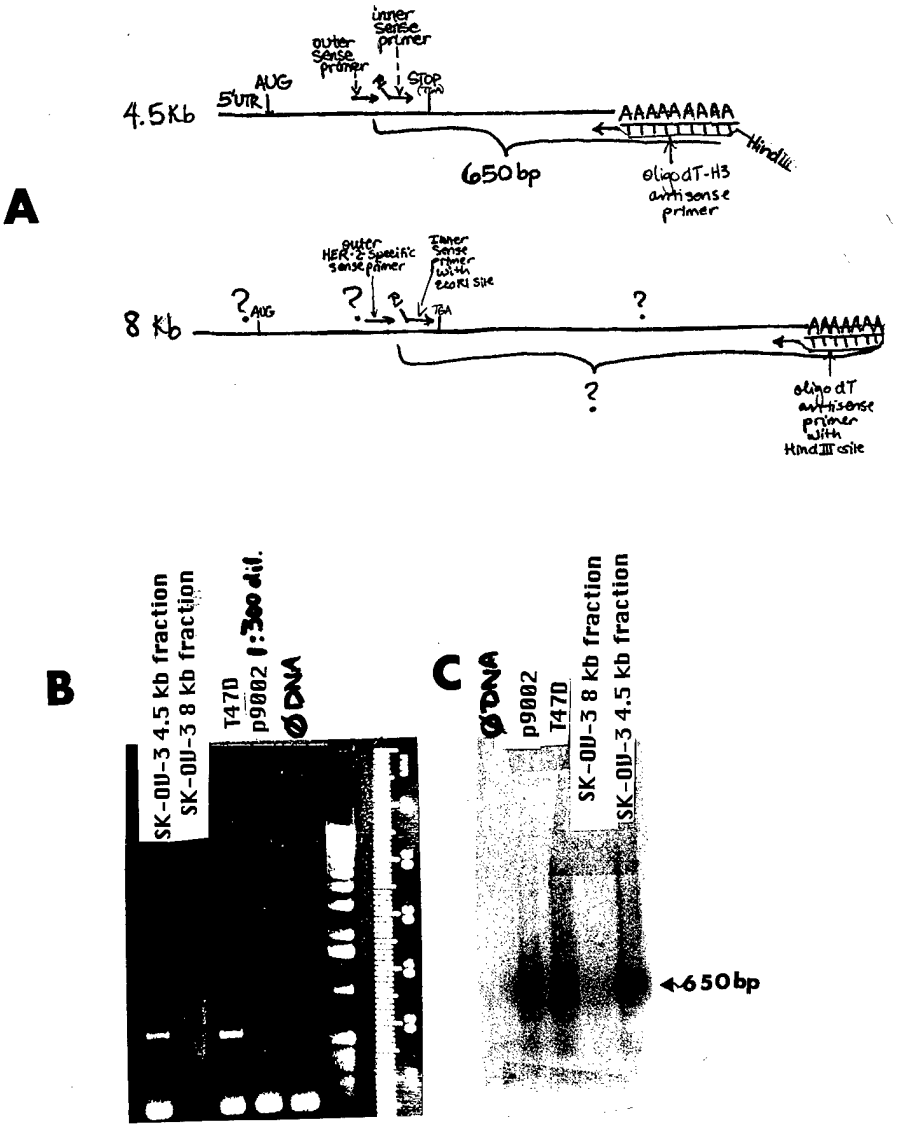


Figure 3. **3'RACE PCR on gel-fractionated, oligo-dT-primed reverse transcribed SK-OV-3 4.5 kb and 8 kb mRNA suggests a possible difference in the 3'UTR size and/or sequence of the larger 8 kb HER-2/neu transcript.** ***A***: Schematic of nesting 3'RACE strategy and expected 650 bp amplification product from the 'wild-type' transcript. ***B***: Representative ethidium bromide-stained, UV-illuminated 0.8 % agarose gel showing electrophoresed products from nesting 3'RACE PCR on: no DNA control, p9002 HER-2 expression vector, T47D cDNA, the 8 kb cDNA fraction from SK-OV-3, and 4.5 kb cDNA fraction from SK-OV-3. ***C***: Southern blot of the gel shown in ***B***, demonstrating hybridization of 650 bp amplification products with a γ(32P)dATP-labelled oligonucleotide specific to HER-2 sequence within the region of amplification. Note that no specific products were obtained from the 8 kb cDNA fraction, indicating a possible variation in the 3'UTR of the 8 kb HER-2 transcript.

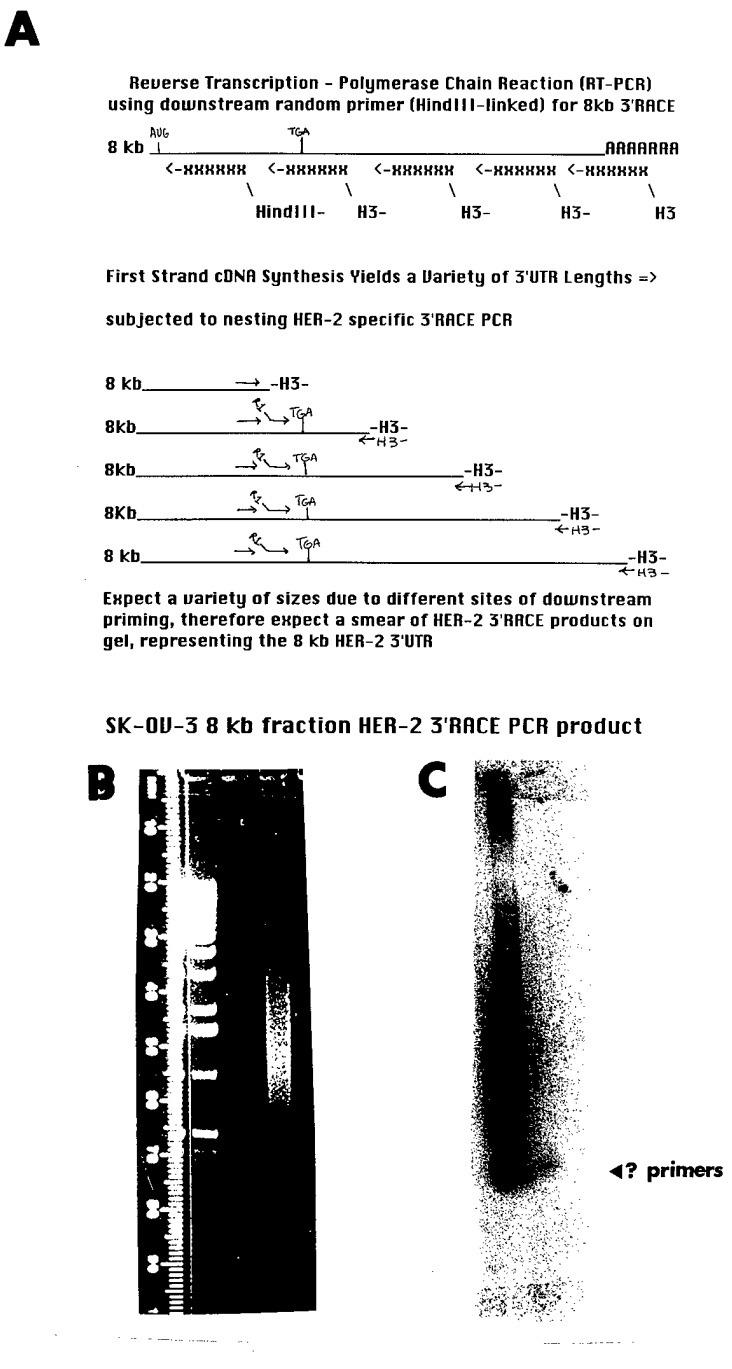


Figure 4. **RT-PCR and 3'RACE strategy on 8 kb mRNA from SK-OV-3** is schematicized in ***A***. ***B***: Representative ethidium bromide-stained, UV-illuminated 1 % agarose gel showing electrophoresed products from nesting 3'RACE PCR on the 8 kb fraction from SK-OV-3. ***C***: Southern blot of the gel in ***B***, showing hybridization with products ranging in size from a few base pairs to approximately 4 kb, as expected, and consistent with a larger 3'UTR in the 8 kb transcript than in the 4.5 kb transcript.

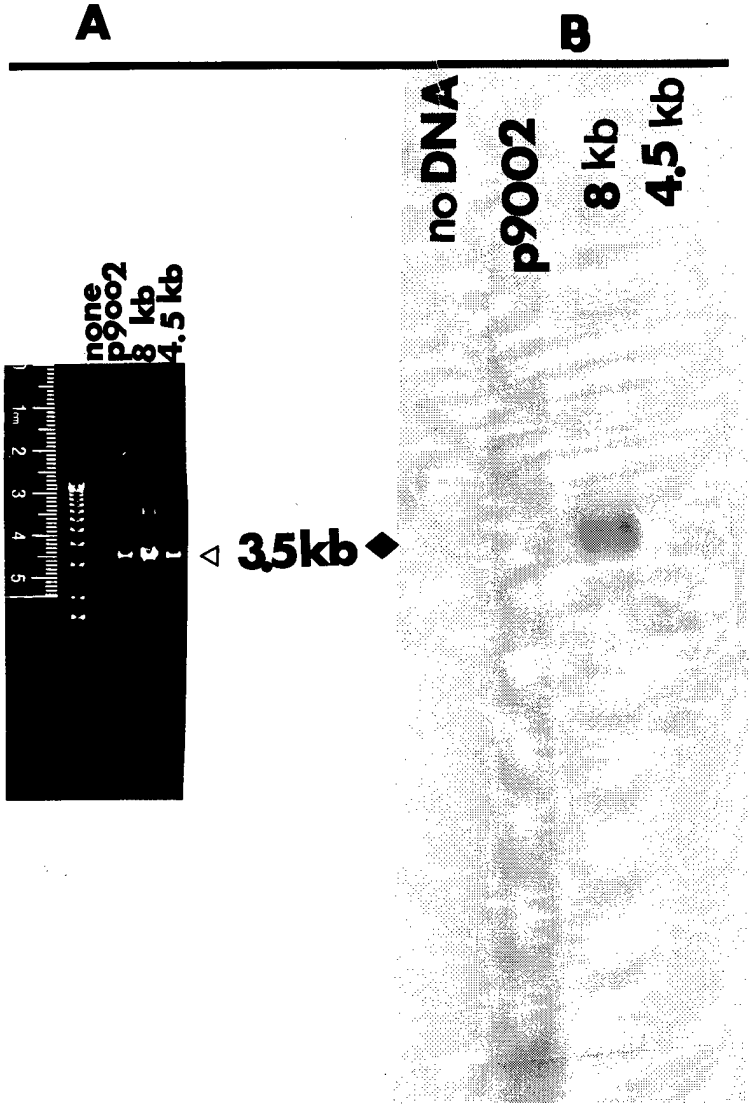


Figure 5. **PCR amplification of 3.5 kb coding region from HER-2/neu cDNAs**. ***A***: Nested PCR products electrophoresed on 0.8% agarose, ethidium bromide-stained and UV-illuminated, showing 3.5 kb amplification products from p9002, 8 kb, and 4.5 kb SK-OV-3 cDNA fractions, with no product in the no DNA control lane. ***B***: Southern blot of the gel in ***A*** showing hybridization of the major 3.5 kb band with a $\alpha(^{32}P)$dCTP-labelled HER-2 cDNA probe.

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